PURIFICATION AND CHARACTERISATION OF PHOSPHORYLASE FROM MUSCLE OF TILAPIA *(TILAPIA MOSAMBICA)*

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Phosphorylase from muscle of tilapia (*Tilapia mosambica*) was extracted by water and purified by ammonium sulphate precipitation, centrifugation and repeated recrystallisation. Electrophorogram of the enzyme preparation showed a single band near origin. The enzyme showed optimum pH and temperature at 6.1 and 37°C respectively. Glucose and glucose-6-phosphate were found to be competitive inhibitors of the enzyme. Maltose and starch acted as primers for the phosphorylase reaction like glycogen.

INTRODUCTION

Fish muscle resembles mammalian muscle in many respects. Muscle glycogen is an important source of energy for fish during muscular activity when glycogen is degraded to lactic acid via the EMP pathway (Burt, 1961 and 1966; Burt and Stroud, 1966; Tarr and Leroux, 1962 and Nagayama, 1961). Fish muscle contains the enzymes required to bring about this oxidative degradation as in mammalian muscle. Fish muscle is a source of both glycogen and fat of which glycogen is used mainly for muscular activity as is evident from the depletion of glycogen in struggled fish (Edith Gould, 1965) while fat is used mainly during spawning season and starvation. In insects, glycogen is used only for flight initiation and then changes over to fat for prolonged muscular activity (Opie and Newsholme, 1967 and Applebaum and Schlesinger, 1973). This is in contrast to the predominant carbohydrate utilisation in mammals where fat is a fuel reserve to be used during starvation.

The enzyme phosphorylase involved in reversible conversion of glucose-1-phosphate to glycogen has been studied by Burt (1966) in crude extracts of cod and by Crabtree and Newsholme (1972) in certain fishes, crustaceans and frog. So far, there is no account of purification and characterisation of muscle phosphorylase from fish. Many compounds are known to affect phosphorylase with vari-

ations depending upon the source of enzyme. The present paper reports the results of experiments carried out to purify and characterise muscle phosphorylase of tilapia.

Materials And Methods

Glycogen, Glucose-1-phosphate dipotassium salt, glyceraldehyde-3-phosphate dipotassium salt and glucose-6-phosphate dipotassium salt (L. Light and Company, England) and adenosine monophosphate (AMP) (Fluka, Switzerland) were repeatedly recrystallised from ethanol water mixture before use, except glycogen, to free from traces of inorganic phosphate and the purity was confirmed by paper chromatography. The reagents for polyacrylamide gel electrophoresis were the products of BDH, England except tris hydroxymethyl amino methane which was a product of Fluka. All other chemicals were of analar quality. A refrigerated high speed centrifuge (International Equipment Company, Model HR-1) was used for centrifugation. Evelyn colorimeter (Honeywell Rubricorn Instruments) was used for colorimetric measurements.

Tilapia weighing 300 to 500 g. caught from fish farms at Malampuzha and Chittoor and maintained live in aquarium tanks were used. Both male and female fish were used indiscriminately.

Purification of the enzyme

Fish were caught and killed immediately by a blow on the head to avoid struggling as far as possible. They were skinned and the lateral muscle excised and 500 g. muscle used for the study. All operations, unless otherwise stated, were carried out in a cold room maintained at 0 to 4°C. Phosphorylase was then extracted according to the method of Cori (1955), unless otherwise specified. The buffer used for dialysis had the composition of 25 ml. 0.015M. ethylene diamine tetra acetic acid (EDTA) and 1000 ml. 0.02 M. Sodium β glycerophosphate and the mixture diluted with distilled water to 2500 ml. and pH adjusted to 6.8. The solvent for the enzyme crystals was composed of 2 ml. 0.0375 M. EDTA and 48 ml. of 0.02 M. sodium β glycerophosphate having a pH 6.8. All pH manipulations were effected with 0 05 N hydrochloric acid to the acidic side and saturated potassium bicarbonate solution to the alkaline side. The dialyses were conducted in cellophane bags 1.3 cm. diameter at 0 to 4°C.

Enzyme assay

The enzyme activity was assayed in the direction of glycogen synthesis as described by Crabtree and Newsholme (1972). The assay medium contained 32m.M. glucose-1-phosphate, 0.5 m. M. AMP,

Table I

SUMMARY OF PURIFICATION

Fraction	Total activity	Sp. activity	Percentage of recovery
Crude	13,200	0.0878	—
First crystals	8,700	25.87	69.9
Second crystals	5,525	35.65	41.5
Third crystals	4,520	38.39	34.3

2% (w/v) glycogen and pH adjusted to 6.2. The reaction was initiated by the addition of 0.2 ml. of the enzyme preparation to 0.2 ml. of the assay medium in a polystyrene tube kept incubated at 36°C for 15 minutes. The reaction was terminated by the addition of 0.6 ml. perchloric acid. The protein precipitated was removed by centrifugation and in the centrifugate the inorganic phosphate liberated was estimated by the method of Fiske and Subbarow (1925). The assay is based on the assumption that during this reaction the liberation of inorganic phosphate is linear. AMP and glucose-1-phosphate were omitted from the control. Protein concentrations were determined by the method of Follin and Ciocaltue (1927).

Results

Purification

Table I shows the summary of purification of tilapia muscle phosphorylase. An enzyme unit is defined as the amount of enzyme in mg. that liberates one micromole of inorganic phosphate per minute under optimum conditions of temperature and pH. Specific activity is defined as the number of units of enzyme per mg. of protein.

Effect of pH

The effect of pH was studied by carrying out the phosphorylase reaction for 15 minutes in 1 ml. citrate buffer as given in assay methods and then merging the results with those from trismaleate buffer. Muscle phosphorylase of tilapia recorded only one pH optimum at 6.1 (Fig. 1) as against two pH optima at 5.5 and 6.7 for cod muscle phosphorylase

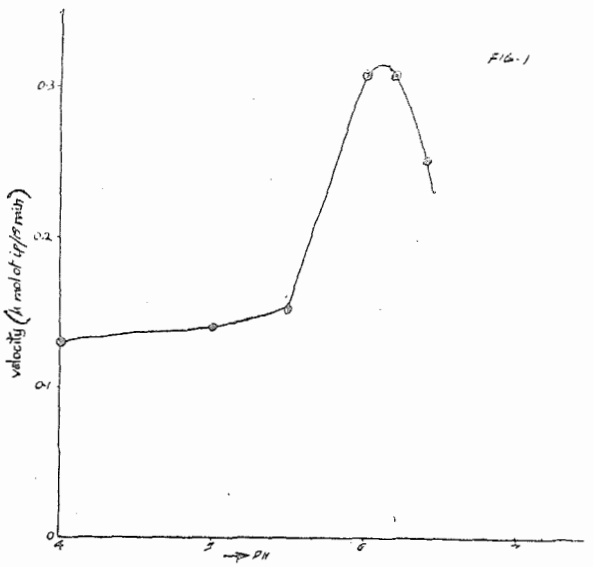

Fig. 1

Effect of pH on Tilapia muscle phosphorylase activity. The assay conditions were as described under materials and methods.

(Burt, 1966), 6.2 for mammalian muscle phosphorylase (Sumner and Myrback, 1951) and 6.7 for fat body phosphorylase of locusts (Applebaum and Schlesinger, 1973).

Effect of temperature

The reactions were carried out at temperatures varying from 20 to 55°C for 15 minutes. The activity showed the maximum value at 36°C, almost similar to mammalian enzyme. The enzyme lost most of its activity above 50°C. These results are indicated in Fig. 2.

Substrate specificity

For study of substrate specificity, 0.2 ml. each of saturated solutions of glucose-1-phosphate and glucose-6-phosphate were separately incubated at 36°C with 0.2 ml. of a solution containing 2%

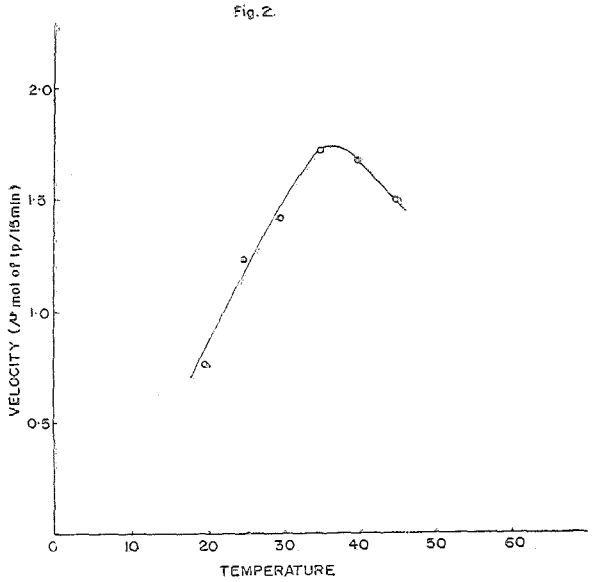

Fig. 2

Effect of temperature of Tilapia muscle phosphorylase. The assay conditions were as described under materials and methods.

glycogen and 0.5 m. M. AMP for 30 minutes. At the end of 30 minutes, the reaction mixture was heated to 50°C to inactivate the enzyme. Then the reaction mixtures were chromatographed on paper along with standards for glucose-1-phosphate, glucose-6-phosphate, AMP and dipotassium hydrogen phosphate. They were run with butanol : acetic acid : ethanol : water in the proportion of 120 : 50 : 25 : 25 for 20 hours and developed with phosphate reagent (Ivor Smith, 1960). Inorganic phosphate was released only from the reaction mixture that contained glucose-1-phosphate showing that tilapia muscle phosphorylase is specific for L-D. glucose-1-phosphate. Studies were also carried out in a similar way to see the nature of primers to which tilapia muscle phosphorylase can add on glucose units. The results indicated that apart from glycogen, maltose and starch had the capacity to act as primers, but not dextrin.

Inhibition

Glucose and glucose-6-phosphate are competitive inhibitors of tilapia muscle phosphorylase for the substrate glucose-1-phosphate. The rate of inhibition varied with increase in concentration of substrate in both cases. AMP relieved the inhibition by glucose-6-phosphate as recorded by Kamagawa and Fukui (1971) in disagreement with the report of Applebaum and Schlesinger (1973) (Fig. 3).

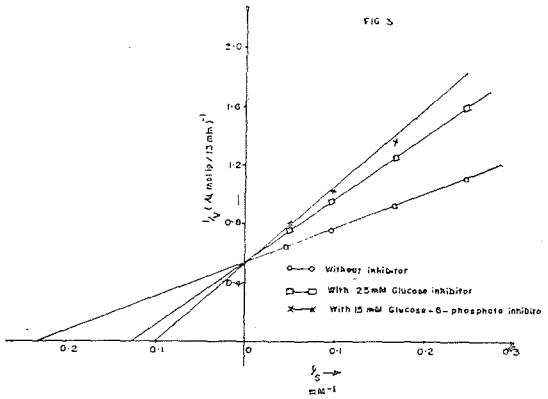

Fig. 3

The effect of inhibitors, glucose and glucose-6-phosphate on tilapia muscle phosphorylase

- 0 without inhibitor
- □ with 25 m.M. Glucose inhibitor
- x with 15 m.M. Glucose-6-phosphate inhibitor

Assay conditions as given under materials and methods

Effect of primer concentration

The velocity of tilapia muscle phosphorylase for a given concentration of enzyme increased with the increase in

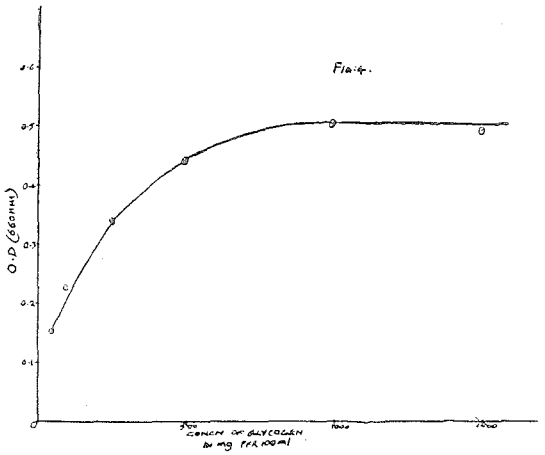

Fig. 4

Influence of glycogen concentration on phosphorylase reaction. Assay conditions as given under materials and methods. Specific activity of the enzyme 3.839.

concentration of glycogen. For an enzyme preparation with specific activity 38.39 the maximum activity was recorded for a glycogen concentration of 1g. per cent. Further increase in glycogen concentration had no effect on the velocity of phosphorylase reaction. (Fig. 4).

Discussion

The function and distribution of glycolytic enzymes are technologically important. The fact that the breakdown products of carbohydrate contribute to flavour, taste and texture of meat in general stresses this point. Further, the enzymes involved in carbohydrate breakdown play an important role in rigor mortis, since the amount of lactic acid formed during the premortem activity influence the time for its onset and duration, (Tomlinson, Jonas and Geiger, 1963 and Burt, 1966) along with adenosine triphosphate concentration.

Tilapia muscle phosphorylase is similar to mammalian muscle in several respects. The optimum temperature and pH are almost identical to the mammalian enzymes reported by Cori (1955). Though AMP activates fish muscle phosphorylase, it is not essential for fresh preparations of the enzyme, showing the existence of active 'a' and inactive 'b' forms of phosphorylase in tilapia muscle as in cod (Burt, 1966 a) and mammals (Cori, 1955). In this respect tilapia muscle phosphorylase is different from protozoan and yeast phosphorylase whose activity is fully independent of the presence or absence of AMP (Applebaum and Schlesinger, 1973). The extent of inhibition by glucose and the capacity of AMP to relieve it forms another similarity of tilapia muscle phosphorylase to mammalian muscle phosphorylase. Above all, like phosphorylases from all sources tilapia muscle phosphorylase is specific for the substrate L-D-glucose-1-phosphate. The main difference between mammals and tilapia as regards the enzyme content is its three fold content in the former than in the latter. But when compared to insects and crustaceans (Opie and Newsholme, 1972 and Applebaum and Schlesinger, 1973) tilapia contains a larger proportion of the enzyme. Thus tilapia is in between mammals, and insects and crustaceans, when the extent of glycolysis is considered.

The inhibition by glucose and glucose-6-phosphate explains the preferred use of them over the polysaccaride glycogen, which are comparatively a quick source of energy in glycolysis. The inhibitory effects of these compounds will be transcient *in vivo* due to its oxidation in EMP pathway, that it can only be a case of product inhibition. Thus intermediates

of glycolytic pathway cannot act as a standing inhibitor *in vivo*. So, for any attempt to suppress glycolysis an end product of the carbohydrate metabolism will be of better use than the glycolytic intermediates.

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